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Influence of an Antiviral Compound on the Temperature Dependence of Viral Protein Flexibility and Packing: a Molecular Dynamics Study

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The antiviral activity of compounds that bind an internal pocket of picornaviruses is due in part to stabilization of the protein capsid and inhibition of the uncoating process required for virus replication. Information on the basis for this structural stabilization of the virus capsid is important to elucidate the mechanism of antiviral action and provide insights into the disassembly process. It has been proposed that this stabilization is entropically based, since binding the nonpolar antiviral compound increases the compressibility, and thus the conformational flexibility, of the virus. Such a proposal predicts a difference in the temperature dependence of the atomic positional fluctuations for free virus and drug-bound virus; nonpolar interactions are weaker and less directional, and would give rise to greater conformational disorder at low temperature. Further, the transition that has been observed in globular proteins to a state resembling a frozen liquid, in which the protein is considered ''trapped'' in potential energy wells, is predicted to occur at lower temperature when the antiviral compound is bound. Results described here from computer simulations of rhinovirus over a range in temperature show these predicted changes in conformational disorder and the temperature of the transition in mobility. In addition to providing independent support for the above proposal for antiviral activity, these results indicate that the mobility transition of a protein can be controlled by the binding of an appropriate ligand, an effect not previously reported.

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Antiviral compounds bind to an internal pocket of the icosahedral protein capsid of picornaviruses (Smith *et al*., 1986; Rossman, 1994; Grant *et al*., 1994), including human rhinovirus protein and poliovirus. In some cases, the antiviral activity of these compounds derives from structural stabilization of the assembled viral capsid (Fox *et al*., 1986; Rombaut *et al*., 1991; Bibler-Muckelbauer *et al*., 1994), thus inhibiting the uncoating process for releasing RNA from the capsid and stopping viral replication. Based on results from recent computer simulation studies (Phelps & Post, 1995), it was proposed that the structural stabilization, and hence antiviral activity, has an entropic, rather than strictly energetic, basis. One consequence of filling the pocket with the antiviral compound was an observed increase in the fluctuations in local density of the capsid protein, indicating a more compressible, less ordered structure. Indeed, an empirical correlation between the compressibility (equivalent to density fluctuations) and entropy was noted for globular proteins (Phelps & Post, 1995). Given that the entropy of the assembled viral coat is raised, it was argued that the free energy driving force for uncoating would be reduced, inhibiting viral disassembly and replication. The increase in density fluctuations for the complex of virus and antiviral compound was traced to the nonpolarity of the antiviral compound. A change in the balance of polar/nonpolar interactions is born out by the differences in the electrostatic and van der Waals energy components for free and bound virus. The time-average values determined from the simulations for interactions between protein atoms lining the pocket and all other atoms indicate diminished electrostatic inter-

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actions for HRV14·WIN52084 s relative to HRV24 (−297 *versus* −340 kcal/mol, respectively) and a somewhat more favorable van der Waals energy (−188 *versus* −177 kcal/mol, respectively), to give overall a more nonpolar environment. The reduced structural order in the folded protein can be understood based on the fact that the nonpolar interactions with the antiviral compound are weaker than the protein–protein and protein–solvent ones for which they substitute.

This earlier proposal that the viral protein when bound with the antiviral agent possesses a higher conformational entropy, leads to the predication of an alternative reflection of these changes which is testable both computationally and experimentally. Substitution of strong, polar interactions in the free virus for weaker dispersive ones, with less directionality when the antiviral compound binds, is equivalent to a change in the conformational energy from a rugged surface with high potential energy barriers to one with lower barriers. Figure 1 characterizes the contrast in energy surfaces suggested between free virus (Figure 1A) and the virus complex (Figure 1B). If the earlier interpretations are correct, one should also expect binding to induce an increase in conformational disorder at low temperature consistent with the disparate energy surfaces, and a lowering of the transition temperature T_g in particular. Here T_g refers to a transition in the temperature dependence of the atomic thermal fluctuations of proteins. Neutron scattering studies on globular proteins (Doster *et al.*, 1989) have demonstrated this transition, whereby the conformational mobility of the system becomes kinetically trapped in local energy minima near T_g. The planes in Figure 1C and D depict a temperature near T_g, and illustrate the basis for the predicted lowering of T_g upon binding of the antiviral compound. The temperature at which conformational fluctuations become limited to single wells is lower in Figure 1D because of the smoother surface and lower barriers from weaker forces. T_g has been described as a glass transition temperature (Doster *et al.*, 1989), but is perhaps more

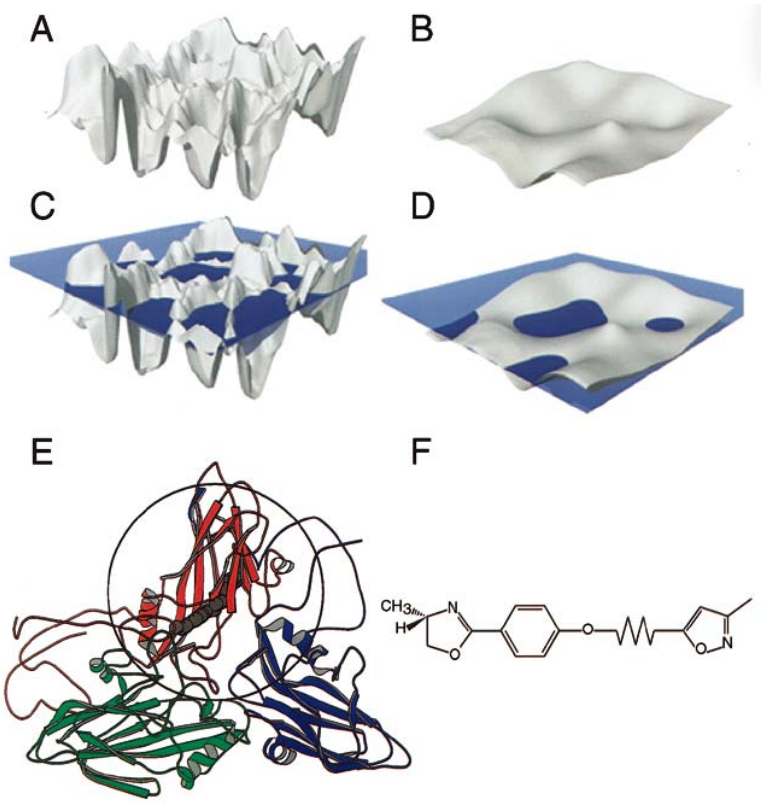

Figure 1. Schematic drawings to represent conformational energy surfaces for HRV14 and HRV14·WIN52084s. A, High energy barriers correspond to the comparatively strong interatomic interactions in HRV14. B, Lower energy barriers and a smoother surface correspond to the weaker interatomic interactions from binding the nonpolar drug for the HRV14·WIN52084s complex. Greater conformational disorder arises from increased accessibility. The difference in T_g for these two surfaces is illustrated by the planes in C and D. The plane, representing the temperature at which the system becomes trapped in energy wells by cooling and lowering the energy of thermally accessible states, is higher for a rugged surface, C, than a smooth one, D. E, Ribbon drawing of the HRV14 asymmetric unit comprising viral proteins VP1 (red), VP2 (green), and VP3 (blue) with a space filling model of WIN52084s (VP4 not shown). The circle indicates the boundary of the spherical simulation region. All trajectories were calculated with the academic version of CHARMM 22 (Brooks *et al.*, 1983), using the CHARMM 19 polar hydrogen parameters and TIP3P model for water molecules. The hydrogen-heavy atom and water-molecule bonds were held fixed with SHAKE (Ryckaert *et al.*, 1997). The simulation region for stochastic-boundary molecular dynamics (Brooks & Karplus, 1983) was a 22 Å radius sphere, centered about the WIN52084s binding site, which included protein atoms from the primary asymmetric unit (shown here) and from icosahedrally related units, crystallographically defined water molecules and modeled water molecules. To model water molecules, the crystallographic coordinates were overlayed with a thermally equilibrated box of water molecules, and those within van der Waals contact of crystallographic atoms were deleted, leaving 359 or 309 water molecules to solvate HRV14 or HRV14·WIN52084s, respectively. The energy was minimized and the system equilibrated thermally for 100 ps, followed by an 800 ps simulation period for analysis. Additional details of the methodology are described elsewhere. F, Chemical structure of the antiviral compound WIN52084s. (A to D generated with the Bryce2 program, and E with MOLSCRIPT (Kraulis, 1991).)

appropriately considered simply as a mobility transition temperature, since the relaxation of proteins do not conform to the canonical definition of a glass-forming system (Green *et al*., 1994; Angell, 1995). T_g is controlled by two related characteristics of a system, the degree of molecular order and strength of interatomic forces (Donth, 1992); an increased range of accessible molecular configurations or decrease in the strength of interatomic interactions favors a resistance to such trapping and a lowering of T_g. Correspondingly, it has long been recognized that so-called free volume plays an important role in determining the onset of T_g (Fox & Flory, 1950; Cohen & Grest, 1979). Free volume, used here to mean the volume of a system in excess of the geometric (i.e. van der Waals) volume of the component atoms, decreases with decreasing temperature, simultaneously lowering conformational mobility. It follows that the mobility transition will occur at a lower temperature for systems with a larger characteristic free-volume above T_g.

In this communication, we explore a mobility transition in human rhinovirus 14 (HRV14) capsid proteins by computer simulation methods, and consider explicitly the effect on T_g of binding an antiviral compound WIN52084s to a hydrophobic pocket in HRV14. T_g is found to be dramatically lower for HRV14·WIN52084s than for HRV14, consistent with the proposal above and also with shifts in T_g resulting from chemical modifications to intermolecular potentials for ''true'' glass-forming systems (Donth, 1992). In addition to providing independent support for the proposal on antiviral activity developed earlier, these results indicate that the mobility transition of a protein can be controlled by the binding of an appropriate ligand, an effect not previously reported. Further, we suggest that measured shifts in T_g associated with ligand binding can be a useful new experimental probe of the activity and mechanism of antiviral agents.

The considerable recent interest in the glass-like behavior of proteins (Kohler *et al*., 1988; Green *et al*., 1994; Angell, 1995; Kuczera *et al*., 1990; Smith *et al*., 1990; Frick & Richter, 1995; Hagen *et al*., 1995) lies partly in its relevance to elucidating the nature of the conformational energy surface and how the properties associated with such a surface relate to protein function. On lowering the temperature of a typical (non-protein) glass below T_g, the system changes from a liquid-like state with considerable conformational mobility to a glassy state in which the structure is that of a frozen liquid. Similarly for proteins, the transition to a ''frozen'' state involves a sharp decrease in conformational freedom. The cessation of significant diffusive motions in the ''frozen'' state hinders functions of the protein that require conformational rearrangement (Ansari *et al*., 1986; Hagen *et al*., 1995). The element common to both conventional glass formers and proteins is that both possess a rugged conformational energy surface with a high density of conformational states separated by large energy maxima (Young & Scholl, 1991). These maxima cause the system to become kinetically trapped in local minima when the temperature is lowered. The microscopic dynamics of these systems has been investigate both by computer simulations methods (Kuczera *et al*., 1990; Loncharich & Brooks, 1990), neutron scattering experiments (Doster *et al*., 1989; Smith *et al*., 1990; Frick & Richter, 1995), and spectral hole-burning techniques (Kohler *et al*., 1988). These earlier results have demonstrated that computer simulation can capture the experimentally observed mobility transition to a ''frozen'' state.

HRV14, one of the causative agents of the common cold, is an icosahedral virus (Rossmann *et al*., 1985) comprising 60 identical asymmetric units (Figure 1E). These spherical viruses are characterized by deep canyons which encircle each viral fivefold axis. Antiviral compounds (Figure 1F) bind to an interior pocket near the base of the canyon, with one binding site per asymmetric unit for a total of 60 sites per virion. The pocket of the virus in the absence of antiviral compounds contains several water molecules, most of which are displaced upon ligand binding. The atomic resolution coordinates for both the unliganded virus coat proteins (PDB entry 4rhv, refined at 3.0 Å resolution) (Rossmann *et al*., 1985) and HRV14 bound to WIN52084s (PDB entry 2rsl, 3.0 Å resolution) (Smith *et al*., 1986) have been determined.

We have examined the influence of the agent WIN52084s on the molecular mobility of HRV14 from a series of molecular dynamics computer simulation trajectories calculated at four temperatures 150, 200, 250 and 300 K for HRV14 and HRV14·WIN52084s. A computationally feasible model for simulating rhinovirus was achieved by employing the stochastic boundary molecular dynamics methodology (Brooks & Karplus, 1983). The 22 Å radius spherical simulation region centered about the WIN52084s binding pocket included 2300 protein atoms primarily from VP1, one of four viral proteins, and 359 or 309 water molecules for HRV14 and HRV14·WIN52084s, respectively. The protein was fully solvated within the simulation sphere, and all trajectories had an 800 ps analysis period. The systems were thermally equilibrated prior to the analysis period during a 100 ps equilibration period, as judged by the temperature stability and deviations in mean positions of main-chain heavy atoms of less than 0.2 Å.

The temperature dependence of two key properties, the mean square fluctuations in atomic position, $\langle \Delta r^2 \rangle$, and % free volume, was followed in order to identify the mobility transition temperature, T_g, for both the unligated and ligated HRV14. For proteins in general, transition to a ''frozen'' state has been experimentally identified from a change in the temperature derivative of $\langle \Delta r^2 \rangle$ by neutron scattering. The measurements (Doster *et al*., 1989) show at temperatures below T_g a notable decrease in $d\langle \Delta r^2 \rangle / dT$ and a loss of temperature dependence in this slope, a characteristic of a harmonic solid, and at temperatures above T_g the fluc-

tuations depend more strongly on temperature as the protein begins to sample multiple energy minima and $d\langle\Delta r^2\rangle/dT$ increases. Computer simulations of proteins (Loncharich & Brooks, 1990; Smith *et al.*, 1990), and more recently of other materials regarded as typical glasses (Angell, 1995), reproduce the observations. Although there is some discussion of whether proteins are ''true'' glasses (Green *et al.*, 1994), as signified by a marked increase in heat capacity or by a non-Arrhenius temperature dependence of viscosity (hence our reference to a mobility transition), it is clear that the corresponding change in $d\langle\Delta r^2\rangle/dT$ reflects a dramatic alteration in internal protein fluctuations which can be understood in the same terms.

For practical reasons, the present study only samples the temperature range coarsely. Nevertheless, when both free volume and atomic fluctuations are considered, the existence and location of a mobility transition can be ascertained. From the results for the mean square fluctuations *versus* temperature, plotted in Figure 2A, T_g is marked by a change in $d\langle\Delta r^2\rangle/dT$ near 250 K for HRV14, while it is near 200 K for HRV14·WIN52084s. A plot of % free volume *versus* temperature, shown in Figure 2B, also supports the appearance of a transition near 250 and 200 K for HRV14 and HRV14·52084s, respectively. The transition at 250 K for HRV14 is well defined for $\langle\Delta r^2\rangle$ (Figure 2B), but less apparent from free volume (Figure 2B), while that at 200 K for HRV14·WIN52084s is weak in Figure 2A, but clearly evident in Figure 2B. The temperature dependence of $3\langle\Delta x^2\rangle$ estimated from neutron scattering data on myoglobin (Mb) (Doster *et al.*, 1989) is also plotted in Figure 2A. The simulated values here behave qualitatively in accord with those measured on this different system. For Mb, the measured data also suggest a mobility transition, with a value of T_g that is intermediate between that of HRV14 and HRV14·WIN52084s.

Increasing hydrophobic character of interatomic contacts is associated with larger linear thermal expansion coefficient, α, due to the relatively weaker interatomic forces involved. Here, α is estimated from the change in free volume with temperature (Figure 2B). We find that above T_g, α is estimated to be 70 and 160×10^{-6} K^{-1} for HRV14 and HRV14·WIN52084s, respectively, consistent with the significant increase in the relative hydrophobicity in the viral complex noted earlier. For comparison, for metmyoglobin, an all-helical protein in which the inter-helical contacts are primarily hydrophobic, α is 115×10^{-6} K^{-1} (Frauenfelder *et al.*, 1987).

The departure of $\langle\Delta r^2\rangle$ at temperatures above T_g from the linearity associated with harmonic fluctuations at lower temperatures has been attributed, in part, to the nonequivalent distribution of $\langle\Delta x^2\rangle$ values for different types of protein atoms (Smith *et al.*, 1990). This departure could also arise from anharmonic contributions to the fluctuations

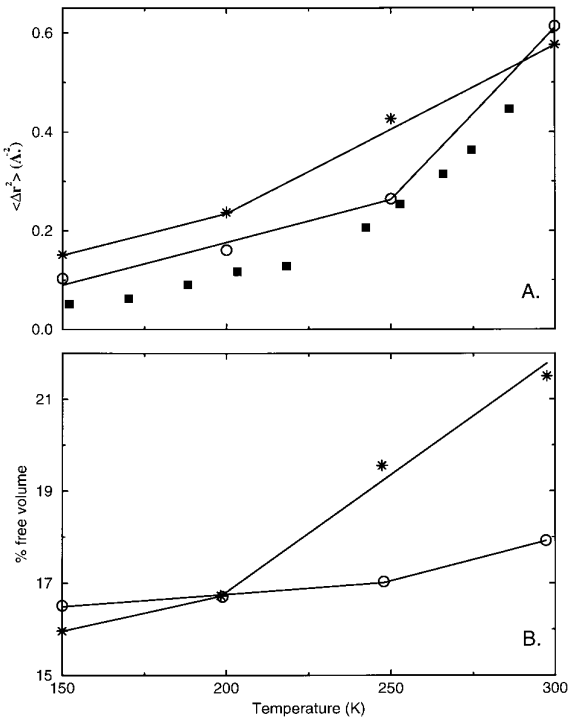

Figure 2. A, Amplitudes of the mean square fluctuations in atomic positions averaged over heavy atoms of HRV14 (○) or HRV14·WIN52084s (*) as a function of temperature. The mean square fluctuations for heavy atoms were calculated from coordinates stored every 0.1 ps over the entire 800 ps of the trajectory. Fluctuation amplitudes calculated for myoglobin from data from Doster *et al.* (1989) are also shown (■). B, Temperature dependence of the time-averaged % free volume. Free volumes were calculated by analyzing grid points in an 18 Å × 18 Å × 10 Å box centered about the drug-binding pocket; the fraction of grid points not lying within the van der Waals radius of any atom defines the fractional free volume, as described earlier (Phelps & Post, 1995). Symbols as in A.

(Smith *et al.*, 1990; Loncharich & Brooks, 1990; Angell, 1995). The detailed analysis permitted by simulations indicates that the latter is, at least, a significant factor. The anharmonic contribution to the fluctuations may be measured by the average of the absolute coefficient of excess kurtosis (Mao *et al.*, 1982; Ichiye & Karplus, 1987). Examination of Figure 3 finds that the anharmonicity rises only gradually with temperature below T_g, but jumps to larger values above T_g. The absolute excess kurtosis values reported by Loncharich & Brooks (1990) for myoglobin show a similar break at T_g. Such behaviour of proteins supports the notion that transition out of the ''frozen'' state is associated with significantly more anharmonic character as more energy minima are explored by the system (Angell, 1995).

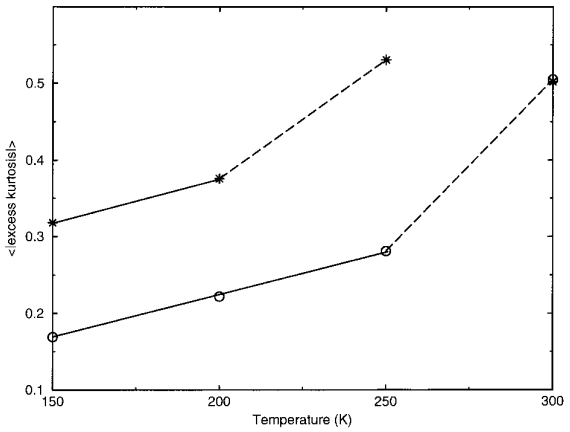

Figure 3. Temperature dependence of the average absolute coefficient of excess kurtosis, $|\langle \Delta x^4 \rangle / \langle \Delta x^x \rangle^2 - 3.0|$; 3.0 is the kurtosis value for a harmonic system. Values are for HRV14·WIN52084s (*) and HRV14 (○).

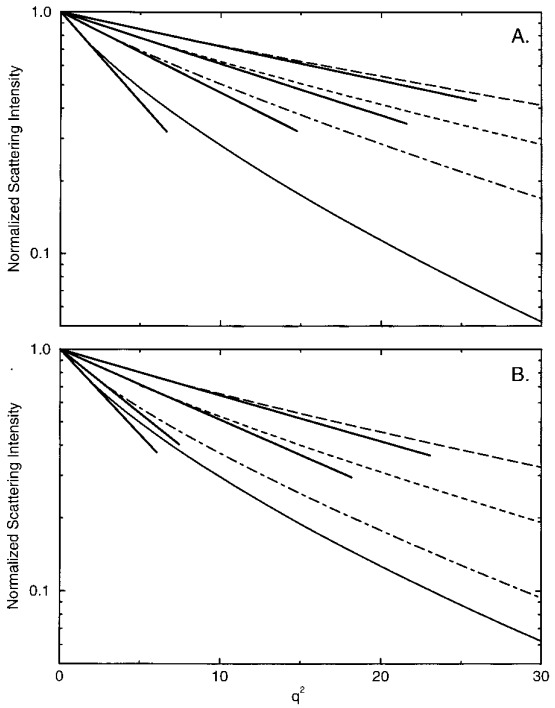

Figure 4. Normalized scattering intensity $S(\mathbf{q}, \omega = 0)$ *versus* q^2, calculated as in Figure 2 of Smith *et al.* (1990) for A, HRV14; and B, HRV14·WIN52084s. Bold lines represent linear behavior based on calculated values of $\langle \Delta x^2 \rangle$ at each T. The curves correspond to increasing temperature from top to bottom: long dash curve, 150 K; short dash curve, 200 K; dot-dash curve, 250 K; and continuous curve, 300 K. We use extended atoms in the simulations and this analysis, rather than the hydrogen atoms that dominate experimental observations. Comparison of the fluctuations of heavy atoms and hydrogen in simulations of myoglobin (Loncharich & Brooks, 1990) indicate that this difference should not be a significant issue here.

Additional evidence that a larger conformational space is, in fact, access ible in the presence of the bound antiviral agent can be found in the dependence of the elastic sca ttering intensity $S(\mathbf{q}, \omega = 0)$ on the scattering wavevector, $\mathbf{q}$. For a Gaussian form, characteristic of non-diffusive harmonic fluctuations, $dS(\mathbf{q}, \omega = 0)/d\mathbf{q}^2$ is constant in the limit $\mathbf{q} \to 0$ and proportional to $\langle \Delta x^2 \rangle$ at that temperature. The curves shown in Figure 4A, calculated (Smith *et al.*, 1990) from the HRV14 simulations are clearly close to linear in $\mathbf{q}^2$ at low temperatures, but show an increasing departure from linearity at higher temperatures (i.e. between 250 and 300 K), indicative of escaping a single potential well. In the presence of WIN52084s, Figure 4B reveals that the departure from linearity at small $\mathbf{q}$ occurs at a distinctly lower temperature (i.e. between 200 and 250 K), confirming the less ordered structure evident in our other results.

We note that at 300 K, HRV14 and HRV14·WIN52084s have nearly equal values of the absolute excess kurtosis coefficient (Figure 3) and similar amplitude fluctuations (Figure 2B), and, thus, elastic scattering curves (continuous curve in Figure 4A and B). Nevertheless, the difference in entropy inferred earlier through the compressibility (Phelps & Post, 1995) continues to distinguish the two states of HRV14 at 300 K as reflected by the free volume (Figure 2B).

The results shown in Figures 2 to 4 consistently manifest a lowering of the apparent T_g of HRV14 upon binding WIN52084s, supporting the picture of increased conformational flexibility induced by the antiviral agent. The literature on (homo-)polymer glasses indicates that the conformational entropy of glass formers is small approaching T_g from above (Donth, 1992). Thus, the relationship between interatomic forces, molecular order and the mobility transition, taken together with our earlier work on the differences in compressibility, strongly support the conclusion that HRV14 has a significantly lower conformational entropy than HRV14·WIN52084s. Others have concluded that some assembled viruses are entropically stabilized (Lauffer *et al.*, 1958; Prevelige *et al.*, 1994; Da Poian *et al.*, 1995), and attribute entropic stabilization to the release of water molecules from apolar sidechains during viral assembly. The overall entropy change associated with viral assembly must be expected to also include a negative contribution from loss of conformational freedom of the proteins involved. Our results indicate that the binding of the antiviral agent WIN52084s to HRV14 has an effect that is thermodynamically inverse to this aspect of the assembly process, imparting greater conformational freedom to the folded state and, thus, greater stability of the assembled HRV14·WIN52084s over HRV14. A similar compensatory effect to the entropy of dimerization of a protein was noted by Tidor & Karplus (1994). Here, binding the antiviral compound changes the

conformational energy surface from a more rugged one, with high barriers (Figure 1A), to a smoother one, with low barriers (Figure 1B), allowing a greater range of conformations to be accessed at lower temperatures. The low-barrier, high-entropy surface (Figure 1B and D) for the weaker, nonpolar interactions in the virus complex stabilizes against disassembly of the virus. Further, if the effects observed for WIN52084s are general for long-chain apolar molecules that occupy the binding pocket, then our results imply a role for the compounds found in the pocket by crystallographic studies, which are thought to bind picornaviruses naturally (Kim *et al*., 1993; Filman *et al*., 1989). Electron density, in the shape of a long-chain fatty-acid molecule, more often than not is found in the region of the pocket for picornaviruses isolated in the absence of antiviral compounds. Our findings support a role for these ''pocket factors'' in the stabilization of the assembled virus particle (Rossmann, 1994; Grant *et al*., 1994) such that steps in viral disassembly would include dissociation of this factor. We conclude that viral uncoating can be controlled by factors which influence the conformational entropy of the folded protein, and that this can potentially be assessed experimentally by low temperature measurements yielding T_g and elastic neutron scattering amplitudes, as well as by scanning calorimetry.

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